ORIGINAL INVESTIGATION

Rot and Agr system modulate fibrinogen-binding ability mainly by regulating *clfB* expression in *Staphylococcus aureus* NCTC8325

Ting Xue · Yibo You · Fei Shang · Baolin Sun

Abstract *Staphylococcus aureus* is an important human pathogen that causes a variety of diseases, ranging from localized skin infections to life-threatening systemic infections. The success of *S. aureus* as a pathogen is partly due to its ability to adhere to a wide range of host tissues by binding to host extracellular matrix proteins such as fibrinogen, fibronectin, and collagen. *Staphylococcus aureus* expresses two proteins that can bind specifically to fibrinogen, clumping factors A and B (ClfA and ClfB). Repressor of toxins (Rot) is known to be a global regulator of virulence gene expression in *S. aureus*. The translation of Rot is regulated by the staphylococcal accessory gene regulator (Agr) quorum-sensing system. In this study, we demonstrated that Rot and the Agr system in *S. aureus* NCTC8325 can affect the bacterial binding ability to human fibrinogen (Fg) under different bacterial growth phases. Our real-time RT-PCR results indicated that both Rot and the Agr system have no significant effect on *clfA* expression. However, Rot is an activator of *clfB*, and Agr/ RNAIII can regulate *clfB* expression via Rot. Gel shift data further suggested that Rot might regulate *clfB* expression by directly binding to the promoter region of *clfB*. Moreover, Rot and the Agr system exhibited consistent regulatory effects on *clfB* transcription and bacterial Fg-binding ability, suggesting that Rot and the Agr system might affect bacterial Fg-binding ability mainly through regulating *clfB* transcription.

Keywords *Staphylococcus aureus* · Rot · Agr quorum sensing · Fibrinogen-binding protein · *clfA* · *clfB*

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Introduction

Staphylococcus aureus is a major community and nosocomially acquired pathogen that can cause both local and systemic infections in humans. A large number of cell-surface-associated proteins and secreted proteins are essential for *S. aureus* pathogenicity [1–3]. Some surface-associated proteins such as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) allow *S. aureus* to bind to host proteins, including fibrinogen (Fg), fibronectin, collagen, and von Willebrand factor, thus enabling the bacterium to colonize and establish a focus of infection [3–6]. *Staphylococcus aureus* can express up to 20 different potential MSCRAMMs that are covalently anchored by sortase to peptidoglycan. Among these, several different proteins can bind specifically to Fg, including clumping factors A and B (ClfA and ClfB). ClfA and ClfB are covalently attached to the cell wall and mediate bacterial adherence to immobilized Fg, blood clots, conditioned biomaterial ex vivo, and thrombin-damaged heart valves in a rat model of endocarditis. The proteins also promote bacterial clumping in the presence of soluble Fg [5, 7–14]. In addition, several secreted proteins of *S. aureus* can bind to Fg, notably coagulase, the bifunctional fibronectin-binding proteins A and B (FnbpA and FnbpB), the extracellular Fg-binding protein (Efb), and MHC class II analogue protein (Map) [7, 15–20].

The most thoroughly investigated virulence regulatory element is the *agr* locus encoding two divergently transcribed transcripts, RNA II and RNA III [21]. The RNA II transcript encodes a two-component signal transduction system, which responds to the extracellular concentration of an autoinducing peptide (AIP) also encoded by RNAII [22, 23]. Induction of this quorum-sensing process results in the production of the 514-nucleotide RNAIII transcript. RNAIII is the actual effector of the Agr system and was, thus, one of the first small regulatory RNAs to be identified in bacteria [24, 25]. However, until recently, the molecular details of how RNAIII regulates expression of a large number of target genes at the level of transcription remained unclear. A major breakthrough occurred when it was shown that RNAIII can inhibit translation of *rot* mRNA by a limited number of base pairings [26, 27]. Rot was first identified in a transposon screening for mutations that could restore proteolytic and hemolytic activities in an *agr*-deficient mutant [28]. Another study confirmed that the *rot* mutation could restore parental virulence of the *agr* mutant in a rabbit model of endocarditis [29]. Therefore, Rot was predicted to function as a global transcriptional repressor of virulence genes, whose activity could be counteracted by RNAIII [30, 31]. Microarray analysis demonstrated that Rot is a negative regulator of many genes encoding extracellular virulence factors [32]. In addition, the array also revealed that Rot might function as a positive regulator of transcription. Noticeably, Rot and Agr system have opposing effects on lots of target genes [31–35].

According to previous studies, it has been accepted that the extracellular protein genes including *entA*, *cna*, and *clfA* are not regulated by *agr* [22]. One study performed in the *S. aureus* NCTC8325-4 indicated that much more ClfB was present in the *agr* mutants than in wild-type cells, suggesting that ClfB expression was negatively regulated by the Agr system. Northern blot analysis further confirmed that *agr* affected *clfB* expression at the level of transcription, as the amount of *clfB* mRNA was significantly increased in *agr* mutant cells [36]. Unfortunately, the mechanism underlying *agr* regulation of *clfB* transcription was not determined. In addition, another study performed in the *S. aureus* Newman suggested that mutation in neither *sarA* nor *agr* affected *clfB* transcription when measured by *lacZ* transcriptional fusions [37], indicating that the impact of *agr* on *clfB* transcription may vary between staphylococcal strains.

It is not clear whether Rot can regulate *clfA/B* expression. According to microarray data, Rot may upregulate several cell surface protein genes including *clfB*, *sdrC*, and *spa* [32]. Oscarsson et al. demonstrated that Rot binds to the promoter region of *spa* and activates its transcription [38]. However, no further work was reported to confirm the activation of *clfB* by Rot.

In this study, we have shown that Rot can activate the bacterial binding ability to human Fg during the bacterial early-exponential phase and the Agr system can repress the binding ability during the exponential phase. We confirmed that Rot and the Agr system did not affect the expression of *clfA*. However, Rot was an activator of *clfB*, and Agr/RNAIII regulated *clfB* expression via Rot. The consistent regulatory effects on *clfB* transcription and bacterial Fg-binding ability mediated by Rot and the Agr system suggested that Rot and the Agr system might affect bacterial Fg-binding ability mainly by regulating the transcription of *clfB*. Furthermore, the transcription of three genes (*efb*, *fnbA*, and *fnbB*) was independent of Agr and Rot, regardless of the growth phase. We also found that the transcription of *map* and *coa* was also regulated by Rot and the Agr system.

Materials and methods

Bacterial strains, plasmids, and growth media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was grown in Luria–Bertani (LB) medium, while the plasmid-containing *E. coli* strains were grown in the same medium but with added antibiotics (ampicillin, 100 mg/1; kanamycin, 50 mg/1). *Staphylococcus aureus* and its derivative strains were grown in LB or TSB (tryptic soy broth, soybean–casein digest medium USP; Oxoid) medium, and when necessary, erythromycin (2.5 mg/l) and chloramphenicol (15 mg/l) were added. The media were solidified with 1.5% (wt/vol) agar, if needed. The mutants were constructed using a method as previously described [39]. For complementing the mutants, the target gene and its promoter from *S. aureus* NCTC8325 were amplified by polymerase chain reaction (PCR). The PCR products were cloned into pLI50 to create complementing plasmids, which were transformed by electroporation into *S. aureus* RN4220, and subsequently transferred to the corresponding mutants. All primers used in this study are listed in Table 2.

DNA manipulation

Genomic DNA of *S. aureus* NCTC8325 was prepared using a standard protocol for gram-positive bacteria [40]. Plasmid DNA from *E. coli* was extracted using a plasmid purification kit (Promega) according to the manufacturer's instructions. Plasmid DNA from *S. aureus* was extracted using the same kit, except that the cells were incubated for at least 10 min at 37°C in the solution of 24 U/ml lysostaphin (Sigma) before the extraction process. The Taq and

Table 1 Strain and plasmid list

Strain or plasmid	Relevant genotype	Reference or source
Strains		
S. aureus		
WT	NCTC8325, wild type	NARSA
RN4220	8325-4, r⁻	NARSA
RN6911	*agr* locus in 8325-4 replaced by tetM	NARSA
SX15	8325 *agr* :: *ermB*	This study
SX16	8325 *agr* :: *ermB* pLIagr	This study
SX17	8325 *rnaIII* :: *ermB*	This study
SX18	8325 *rnaIII* :: *ermB* pLIrnaIII	This study
SX19	8325 *rot* :: *ermB*	This study
SX20	8325 *rot* :: *ermB* pLIrot	This study
SX21	RN6911 *rot* :: *ermB*, *agr/rot* double mutant	This study
E. coli		
DH5α	Clone host strain, *supE44ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1en-dA1 gyrA96 thi-1 relA1*	Invitrogen
BL21	Expression strain, *F⁻, ompT, hsdS (rB⁻mB⁻), gal, dcm (DE3)*	Invitrogen
Plasmids		
pEASY-blunt	Clone vector, Kan^r, Ap^r	Transgen
pET28a(+)	Expression vector	Novagen
pGrot	pET28a(+) with *rot* gene	This study
pEC1	pBluescript derivative. Source of *ermB* gene. Ap^r	R. Bruckner
pBT2	Shuttle vector, temperature sensitive, Ap^r, Cm^r	R. Bruckner
pBTagr	pBT2 derivative, for *agr* mutagenesis; Ap^r, Cm^r, Em^r	This study
pBTrnaIII	pBT2 derivative, for *rnaIII* mutagenesis; Ap^r, Cm^r, Em^r	This study
pBTrot	pBT2 derivative, for *rot* mutagenesis; Ap^r, Cm^r, Em^r	This study
pLI50	Shuttle cloning vector, Ap^r, Cm^r	Addgene
pLIagr	pLI50 with *agr* operon and its promoter, Ap^r, Cm^r	This study
pLIrnaIII	pLI50 with *rnaIII* and its promoter, Ap^r, Cm^r	This study
pLIrot	pLI50 with *rot* and its promoter, Ap^r, Cm^r	This study

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PFU DNA polymerase were obtained from Promega, and the PrimeSTAR® HS DNA polymerase was obtained from Takara. Restriction enzymes were obtained from NEB (New England BioLabs), and the incubation conditions were employed as recommended by the suppliers. *Staphylococcus aureus* was transformed by electroporation as described previously [41].

Total RNA isolation, cDNA generation, real-time RT-PCR, and microarray processing

Overnight cultures of *S. aureus* were diluted 1:100 in LB medium and grown to different growth phases. *Staphylococcus aureus* cells were collected by centrifugation and re-suspended in TE buffer (pH 8.0) containing 10 g/l lysozyme and 40 mg/l lysostaphin. After incubation at 37°C for 5 min, the cells were prepared for total RNA extraction using the Trizol method (Invitrogen), and any residual DNA was removed with DNase (RNase-free, Takara). Real-time RT-PCR was performed with the PrimeScript™ 1st strand cDNA Synthesis Kit and SYBR Premix Ex Taq™ (Takara) using the StepOne™ Real-Time PCR System (Applied Biosystems). The quantity of cDNA measured by real-time PCR was normalized to the abundance of 16S cDNA. The real-time RT-PCR assays were repeated at least three times with similar results.

Purification of Rot

To construct plasmids pGrot used for the overproduction of Rot, the DNA fragment containing the Rot coding region was amplified by PCR from the chromosome of *S. aureus* NCTC8325 using primers g-rot-f-NcoI and g-rot-r-XhoI. The PCR products were digested with NcoI and XhoI and subsequently were ligated with NcoI- and XhoI-digested pET28a(+) (Novagen). The inserts were checked by DNA

Table 2 Oligonucleotide primers used in this study

Primer name	Oligonucleotide (5′–3′)[a]
Up-rot-f-HindIII	GCGaagcttACGTGATAAAGTTAAGGATT
Up-rot-r-SalI	GCGgtcgacTAATAGCATAAAAAGAGGTT
Down-rot-f-BamHI	GCGggatccGCAAAATTCCAAATACAGTG
Down-rot-r-EcoRI	GCGgaattcACACATTTGATTTGTAAGAA
Up-rnaIII-f-BamHI	GCGggatccTTCGTAATTAACGTAAACAG
Up-rnaIII-r-PstI	GCGctgcagCGAAGATAACAAATTTACAA
Down-rnaIII-f-SalI	GCGgtcgacGATTGAAAAAATAACTGTAAAA
Down-rnaIII-r-HindIII	GCGaagcttCTATGTCACGTCTTCAACAT
Up-agrsystem-f-BamHI	GCGggatccGGTATACAAAGAGACGTTACATCA
Up-agrsystem-r-PstI	GCGctgcagGCAGCGATGGATTTTATTTT
Down-agrsystem-f-SalI	GCGgtcgacGATTGAAAAAATAACTGTAAAA
Down-agrsystem-r-HindIII	GCGaagcttCTATGTCACGTCTTCAACAT
Em-f-BamHI	GCGggatccGATACAAATTCCCCGTAGGC
Em-f-NdeI	GCGcatatgGATACAAATTCCCCGTAGGC
Em-f-PstI	GCGctgcagGATACAAATTCCCCGTAGGC
Em-f-HindIII	GCGaagcttGATACAAATTCCCCGTAGGC
Em-r-HindIII	GCGaagcttGAAATAGATTTAAAAATTTCGC
Em-r-SalI	GCGgtcgacGAAATAGATTTAAAAATTTCGC
c-rnaIII-f-BamHI	GCGCggatccAAAATTGAATATGATCTAAGTTATT
c-rnaIII-r-HindIII	GCGCaagcttAATGAATGATTTTCTTAATT
c-agr-f-BamHI	GCGCggatccCGTTAACTGACTTTATTATC
c-agr-r-HindIII	GCGCaagcttAGGAGAGTGGTGTAAAATTG
c-rot-f-BamHI	GCGggatccTAATCATGCTCCATTCATTT
c-rot-r-EcoRI	GCGgaattcTTTAATTTGTTAAATTTAAG
p-clfA-f	GTTGTCGTTTGTAATTCTTAAA
p-clfA-r	CGTGTTTTTCTTTTTTCTTC
p-clfB-f	ACTCCATTTCAATTTCTAGA
p-clfB-r	TGTTGAAATTACAGTAAAATTT
rt-clfA-f	TTTCAACAACGCAAGATA
rt-clfA-r	GCTACTGCCGCTAAACTA
rt-clfB-f	TTTGGGATAGGCAATCATCA
rt-clfB-r	TCATTTGTTGAAGCTGGCTC
rt-coa-f	GCAAATAATTTCGCTAGG
rt-coa-r	CCTTTTCCAACAACCTAT
rt-fnbA-f	ATGATCGTTGTTGGGATG
rt-fnbA-r	GCAGTTTGTGGTGCTTGT
rt-fnbB-f	ACAAGTAATGGTGGGTAC
rt-fnbB-r	AATAAGGATAGTATGGGT
rt-map-f	AAACTACCGGCAACTCAA
rt-map-r	TGTTACACCGCGTTCATC
rt-efb-f	TAACATTAGCGGCAATAG
rt-efb-r	CCATATTCGAATGTACCA
rt-16S-f	CGTGGAGGGTCATTGGA
rt-16S-r	CGTTTACGGCGTGGACTA
g-rot-f-NcoI	GCGccatggTGAAAAAAGTAAATAACGACACT
g-rot-r-XhoI	GCGctcgagCACAGCAATAATTGCGTTTA

[a] The sequences in lowercase letters refer to the restriction endonuclease recognition sites

sequencing. Plasmid pGrot was transformed into *E. coli* BL21 (DE3). The transformant was grown in 1 liter of LB at 37°C to an OD_{600} of 0.3, transferred to 16°C and induced with 0.5 mM IPTG for 3–6 h. Cells were harvested by centrifugation and washed with cell washing buffer (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl). The cells were resuspended in 50 ml of lysis buffer (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl) and lysed by sonication, followed by centrifugation at 12,500 rpm for 30 min at 4°C. The supernatant was mixed with 2 ml of Ni–NTA agarose solution (Invitrogen), and the suspension was loaded onto a column at 4°C. After washing the column with 5 ml of washing buffer I (5 mM imidazole, 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl), 100 ml of washing buffer II (20 mM imidazole, 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl), and then 10 ml washing buffer III (100 mM imidazole, 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl), the Rot protein was eluted with 5 ml of elution buffer (250 mM imidazole, 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 10% glycerol). The imidazole in the eluent was removed using a Centrifuge Biomax-5 column (Millipore), and the Rot protein solution was stored at −80°C until use. The purity of the protein was analyzed by SDS–PAGE, and the protein concentration was measured using the Bradford assay with bovine serum albumin (BSA) as a standard.

Gel shift assay

Two DNA fragments, p-clfA containing *clfA* promoter and p-clfB containing *clfB* promoter, were prepared by PCR from chromosome of *S. aureus* NCTC8325 using two pairs of primers (p-clfA-f, p-clfA-r, p-clfB-f, and p-clfB-r) and PFU DNA polymerase (Promega). The purified DNA fragments were labeled using the digoxigenin (DIG) Gel Shift Kit (Roche) according to the manufacturer's instructions. The labeled fragment was incubated at 25°C for 30 min with various amounts of purified proteins in 10 μl of incubation buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol). After incubation, the mixtures were electrophoresed in a 4% native polyacrylamide gel in a 0.5× TBE buffer. The band shifts were detected and analyzed according to the manufacturer's instructions.

Bacterial adherence to immobilized fibrinogen

Binding of cells to Fg immobilized on plates was measured by the assay from Edihin et al. [11]. Fibrinogen was diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 3.2 μM NaN_3, pH 9.6) to give a range of concentrations from 0.1 to 10 μg ml^{-1}, and 100 μl was used to coat 96-well flat-bottomed ELISA plates (CorStar 3590, Corning) overnight at 4°C. Control wells contained carbonate

buffer only. After washing in 150 mM NaCl with 0.05% Tween 20, the plates were blocked for 1 h at 37°C in 1% BSA and 0.05% Tween in PBS. After washing as before, 100 μl of a cell suspension (OD_{600} of 0.4 in PBS) was added, and the plates were incubated for 2 h at 37°C. After gentle washing by hand, adherent cells were fixed by adding 100 μl of 25% aqueous formaldehyde and incubation at room temperature for at least 30 min. The plates were then washed gently, stained with crystal violet and washed again, and read on an ELX800 microwell plate reader (BioTek) at 560 nm. The assays were repeated six times with similar results.

Results

Rot and the Agr system affect the bacterial binding ability to human fibrinogen when bacterial cells reach different growth phases

To determine whether Rot and Agr affect the bacterial binding ability to human Fg, we performed phenotypic assays in *S. aureus* NCTC8325 and their corresponding mutants. According to previous studies, Rot and Agr have opposing regulatory effects on a large number of target genes, and the masking of *rot* translation by RNAIII might be a key feature of Agr function when *S. aureus* cells grow in the post-exponential phase [22, 26, 42, 43]. Therefore, we tested the Fg-binding ability of these strains when the bacterial cells grew to different cell densities ($OD_{600} = 1$, 2 and 4), representing the different growth phases (early-exponential phase, post-exponential phase, and early-stationary phase). As shown in Fig. 1a, b, when cells reached the early-exponential phase ($OD_{600} = 1.0$), the Fg-binding ability of the *agr* and RNA III mutants showed no significant difference compared with that of the wild type, and the binding ability of the *rot* mutant was lower than that of the wild-type. However, when bacterial cells reached the post-exponential phase ($OD_{600} = 2.0$), the binding abilities of the *agr* and RNA III mutants increased clearly and were much stronger than that of the wild-type and the *rot* mutant. When bacterial cells reached the early-stationary phase, the *agr*, RNA III, and *rot* mutants showed similar binding abilities to that of the wild-type strain (Fig. 1c). These results allow us to conclude that Rot upregulates the Fg-binding ability of the bacterium during the early-exponential growth phase and Agr/RNA III downregulates this ability when cells reach the exponential growth phase. To further confirm the modulatory effect of Rot, we also tested the binding ability of the RN6911 (*agr* mutant) and the RN6911 with *rot* deletion (*agr/rot* double mutant) strains to human Fg when these strains reached different growth phases. As shown in Fig. 1d, e, the binding ability
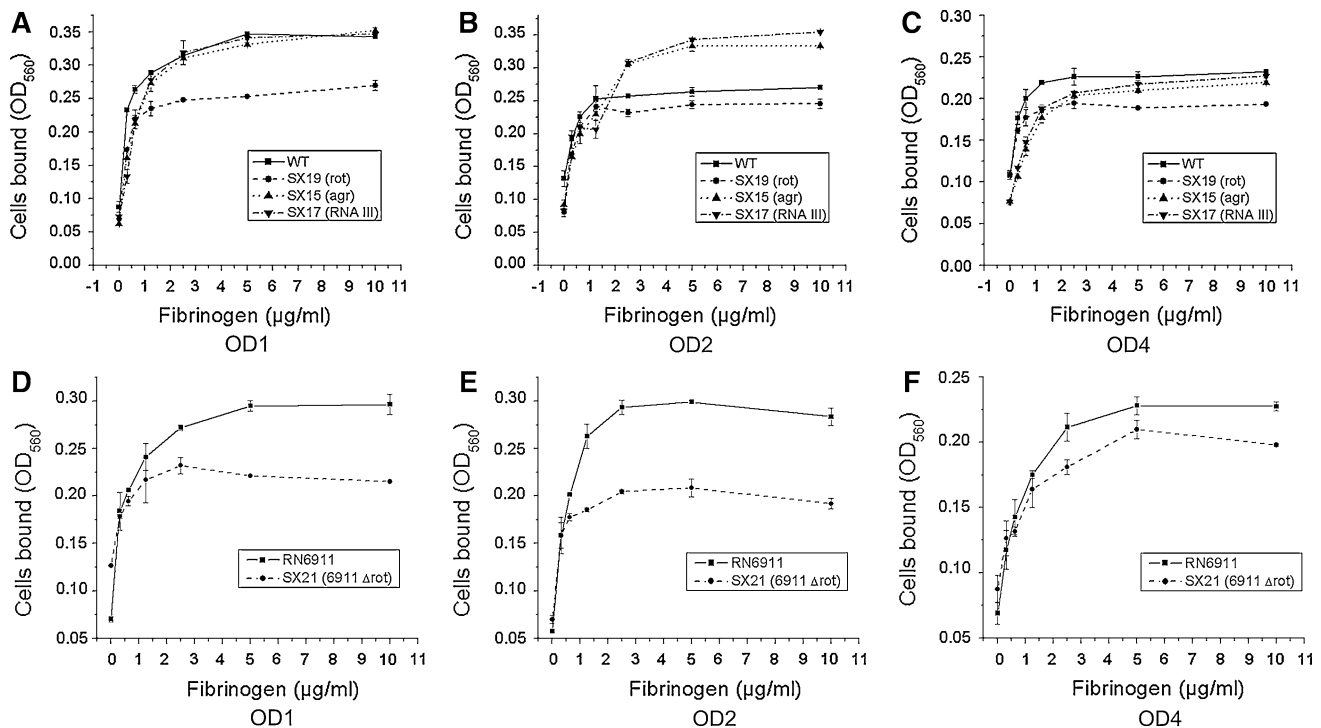
Fig. 1 Influence of Agr, RNA III, and Rot on the bacterial Fg-binding ability. **a–c** Binding ability of WT (*S. aureus* NCTC8325), SX19 (the *rot* mutant), SX15 (the *agr* mutant), and SX17 (the RNA III mutant) to human Fg immobilized on plates was measured. The strains were grown in LB medium to an OD_{600} of 1, 2 and 4.

d–f Binding ability of RN6911 (NCTC8325-4 *agr* mutant) and SX21 (RN6911 *rot* mutant) to human Fg immobilized on plates was measured. The strains were grown in LB medium to an OD_{600} of 1, 2, and 4. The assays were repeated six times with similar results. *Error bars* indicate SD

of the *agr/rot* double mutant was always weaker than that of the *agr* mutant when bacterial cells reach to the early-exponential phase and post-exponential phase, suggesting that Rot can always upregulate the bacterial binding ability to human Fg without the influence of the Agr system. In addition, when comparing the results in Fig. 1b, e, the binding ability of the *agr/rot* double mutant was weaker than that of the wild-type when cells reached the exponential growth phase. This change was similar to that observed with the *rot* mutant but converse to that with the *agr* mutant, indicating that Agr/RNA III might modulate bacterial Fg-binding ability via Rot.

Rot and Agr system have no significant effect on *clfA* expression

According to previous studies, ClfA and ClfB are the main proteins that can specifically bind to Fg, while several other secretory proteins (coagulase, FnbpA, FnbpB, Efb, and Map) have also been shown to bind to Fg but with less ability [7, 15–20]. In order to determine whether the Agr system and Rot regulate the bacterial binding ability to Fg via one or several of these proteins, we performed real-time RT-PCR to measure the transcript levels of the encoding genes of these proteins in the *S. aureus* NCTC8325 and the

corresponding mutants. As described previously, the Agr system does not affect *clfA* transcription in *S. aureus* Newman [37] and NCTC8325-4 [36]. Our results verified this conclusion (Fig. 2a); in addition, we found that the transcript level of *clfA* did not change in the *rot* mutant compared with that in the wild type. To further ensure whether Rot had a modulatory effect on *clfA* expression, we measured the transcript levels of *clfA* in the RN6911 (*agr* mutant) and the RN6911 with *rot* deletion (*agr/rot* double mutant) strains when they reached different growth phases. As shown in Fig. 2b, the transcript level of *clfA* in the *agr/rot* double mutant was almost the same to that in the strain RN6911, suggesting that Rot has no effect on *clfA* transcription. Interestingly, we also performed gel shift assays to check whether Rot can bind to the promoter region of *clfA*, and results showed that Rot displayed a slight binding ability to the *clfA* promoter. However, we did not find this binding ability to contribute to the modulation of *clfA* by Rot (data not shown).

Rot is the activator of *clfB*, and Agr/RNAIII regulates *clfB* expression via Rot

We measured the transcript level of *clfB* using real-time RT-PCR assays, and the results were extremely different
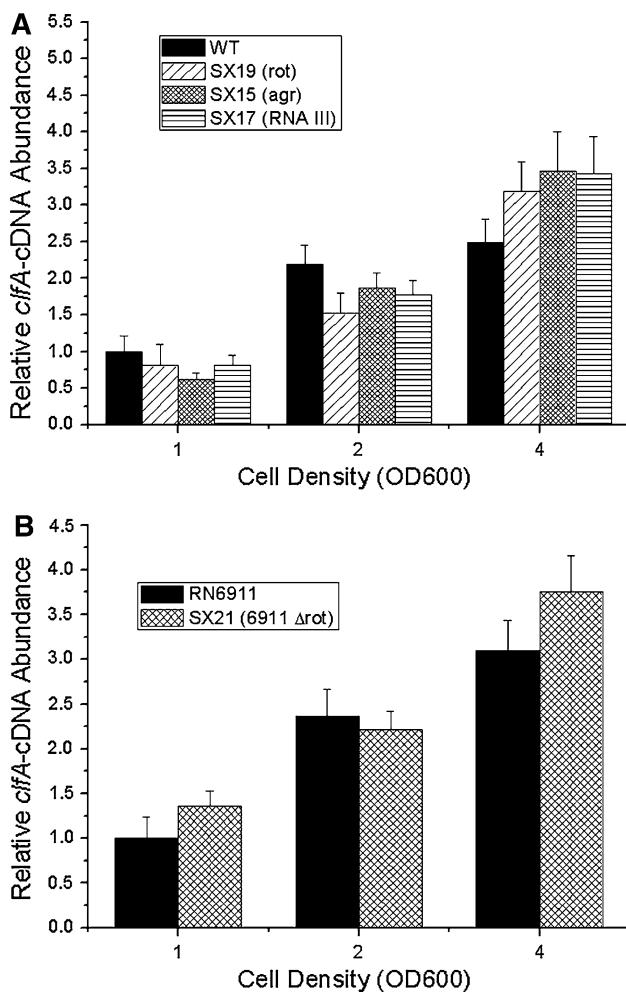
Fig. 2 a The regulatory effects of Rot, Agr, and RNAIII on the transcription of *clfA*. The transcript level of *clfA* was compared using real-time RT-PCR in WT (*S. aureus* NCTC8325), SX19 (the *rot* mutant), SX15 (the *agr* mutant), and SX17 (the RNA III mutant). The strains were grown in LB medium to an OD_{600} of 1, 2, and 4. **b** The regulatory effect of Rot on the transcription of *clfA* without the influence of Agr. The transcript level of *clfA* was compared using real-time RT-PCR in RN6911 (NCTC8325-4 *agr* mutant) and SX21 (RN6911 *rot* mutant). The strains were grown in LB medium to an OD_{600} of 1, 2, and 4. The real-time RT-PCR assays were repeated four times with similar results. *Error bars* indicate SD

from that with *clfA*. As shown in Fig. 3a, when bacterial cells reached the early-exponential phase ($OD_{600} = 1$), the transcript level of *clfB* in the *rot* mutant decreased by about twofold compared with that of the wild-type strain, whereas the transcript levels of *clfB* in the *agr* and the RNA III mutants showed no significant difference with that of the wild-type strain. However, when cells reached the post-exponential phase ($OD_{600} = 2$), the transcript level of *clfB* in the *rot* mutant was similar to that of the wild-type, but the transcript levels of *clfB* in the *agr* and RNA III mutants all increased by about threefold compared with that of the wild-type. We also made all the complementing plasmids and transferred them into the corresponding mutants, and

all the plasmids could restore the wild-type phenotype. According to these results, we suggest that Rot might act as an activator of *clfB* transcription. The activation of *clfB* by Rot could only be observed before the cells reached the exponential phase but this might have been masked by RNA III when the cells reached the exponential phase. When cells reached the exponential phase, the huge amount of RNA III transcripts could have inhibited the translation of *rot* mRNA, thus inactivating *clfB*. Moreover, we postulate that the reason why Agr/RNA III had no significant effect on *clfB* transcription before bacteria reached the exponential phase is that the transcript level of RNA III was lower in the early-exponential phase. In addition, our gel shift assays confirmed that Rot showed high binding ability to the promoter region of *clfB* (Fig. 3b), suggesting that Rot might modulate *clfB* expression by binding to its promoter region. Moreover, as shown in Fig. 3c, the transcript level of *clfB* in the *agr/rot* double mutant was similar to that of the *rot* mutant but was always lower than that of the RN6911 (the *agr* mutant) strain, indicating that Agr/RNA III might regulate *clfB* expression via Rot. It is interesting that the regulatory effect of Rot or the Agr system on *clfB* correlated with that on bacterial Fg-binding ability of Rot or the Agr system. These results lead us to deduce that Rot and the Agr system might affect bacterial Fg-binding ability mainly through regulating the transcription of *clfB*. Furthermore, previous studies revealed that there exists a sigma factor B-dependent promoter upstream of *clfA* and the transcription of *clfA* was positively influenced by sigma factor B [44–46]. Since *S. aureus* NCTC8325 is defective in the sigma factor B [47], we speculated that the transcription of *clfA* might be weakened in this strain. We compared the transcript level of *clfB* with that of *clfA* in the wild-type strain using real-time RT-PCR, and the results showed that the transcript level of *clfB* was higher than that of *clfA*, regardless of the growth phase (Fig. 3d). These results suggest that ClfB might be a more important Fg-binding protein in *S. aureus* NCTC8325 unless its function is masked by ClfA.

Regulatory effects on gene expression of other fibrinogen-binding proteins by Rot and the Agr system

Except for ClfA and ClfB, several other secretory proteins (coagulase, FnbpA, FnbpB, Efb, and Map) can also bind to Fg, albeit to a lesser extent. We performed real-time RT-PCR to measure the transcript levels of these genes in *S. aureus* NCTC8325 and the corresponding mutants. According to our results, the transcription of the three genes (*efb*, *fnbA*, and *fnbB*) was independent of Agr and Rot, regardless of the growth phase. We showed that the transcription of *map* and *coa* was regulated by Rot, and the transcription of *map* was also regulated by Agr system. As
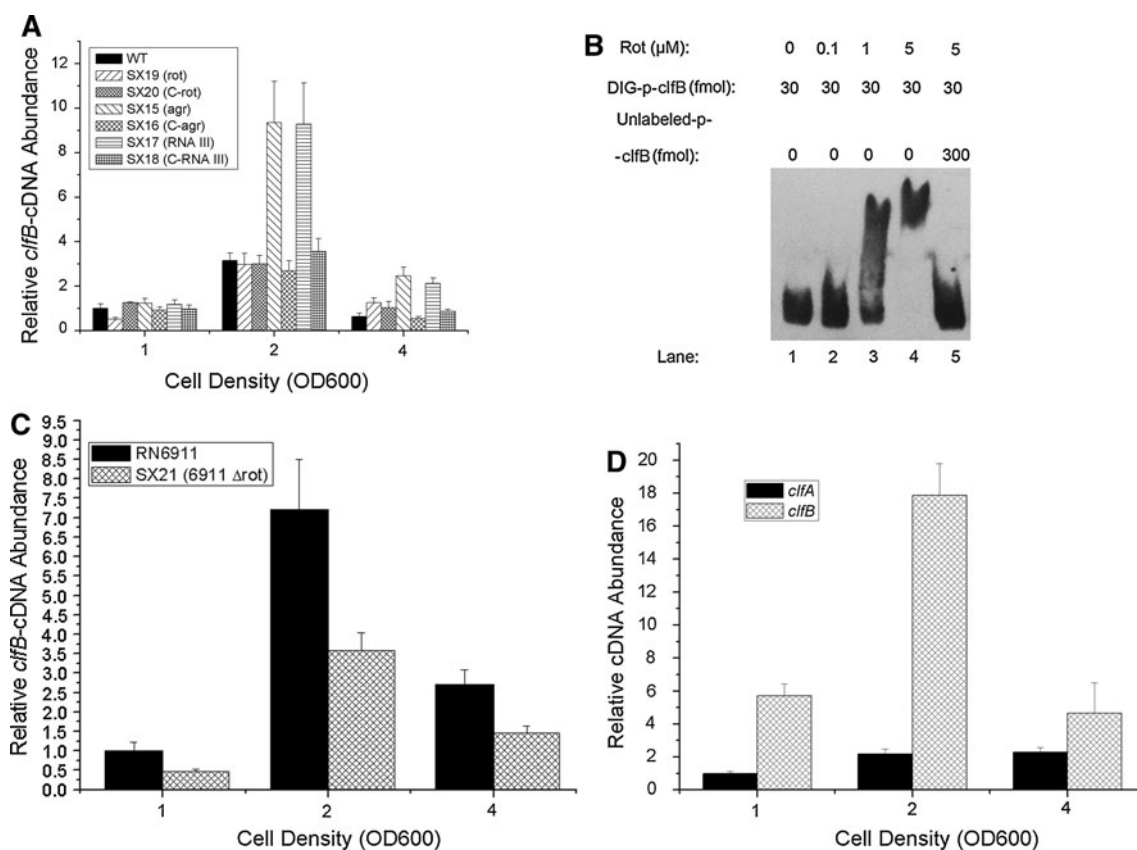
Fig. 3 a The regulatory effects of Rot, Agr, and RNAIII on the transcription of *clfB*. The transcript level of *clfB* was compared using real-time RT-PCR in WT (*S. aureus* NCTC8325), SX19 (the *rot* mutant), SX20 (the *rot* mutant with the complementing plasmid), SX15 (the *agr* mutant), SX16 (the *agr* mutant with the complementing plasmid), SX17 (the RNA III mutant), and SX18 (the RNA III mutant with the complementing plasmid). The strains were grown in LB medium to an OD_{600} of 1, 2, and 4. **b** The binding ability of Rot to the *clfB* promoter as determined by gel shift assays. **c** The regulatory effect of Rot on the transcription of *clfB* without the influence of Agr.

The transcript level of *clfA* was compared using real-time RT-PCR in RN6911 (NCTC8325-4 *agr* mutant) and SX21 (RN6911 *rot* mutant). The strains were grown in LB medium to an OD_{600} of 1, 2, and 4. **d** The comparison of the transcript levels of *clfA* and *clfB* when the bacteria grow to different growth phase. The transcript levels of *clfA* and *clfB* were measured using real-time RT-PCR in WT (*S. aureus* NCTC8325), which was grown in LB medium to an OD_{600} of 1, 2, and 4. The real-time RT-PCR assays were repeated four times with similar results. *Error bars* indicate SD

shown in Fig. 4, the transcript level of *map* was lower in the *agr* mutant and RNAIII mutant compared with that in the wild-type strain, indicating that Agr/RNAIII might be the activator of *map*. In addition, the transcript level of *map* in the SX21 strain (*rot/agr* mutant) was still higher than that in the wild-type strain, suggesting that Agr/RNA III might regulate *map* expression via Rot. Moreover, when bacterial cells reached the early-exponential phase (OD_{600} = 1, Fig. 4a) and post-exponential phase (OD_{600} = 2, Fig. 4b), the transcription of *coa* was activated by Rot. Previous Northern blot assays have shown that the transcription of *coa* was repressed by the Agr system before bacterial cells reached the early-exponential phase (OD_{600} < 1.0) [37]. Furthermore, the transcription was drastically reduced and could not be detected not only in the wild-type strain but also in the *agr* mutant after cells reached the early-exponential phase [37]. Using real-time

RT-PCR, we measured the transcript level of *coa*. Probably because the primers for real-time RT-PCR assays did not work very well, the transcription of *coa* we measured was weak. Besides, we have not observed the regulatory effect of the Agr system on *coa* at the selected time point (OD_{600} = 1, 2, and 4), probably due to the limitation of methodology.

Discussion

During the early phase of infection, *S. aureus* usually secretes a range of cell wall-associated proteins to evade attacks from the host immune system. Some proteins that can bind to host Fg play an important role in bacterial colonization in the host [1–3]. This study indicates that Rot always activates bacterial Fg-binding ability during the

Fig. 4 Influence of Agr and Rot on the transcription of *map*, *efb*, *fnbA*, *fnbB*, and *coa*. The transcript levels of *map*, *efb*, *fnbA*, *fnbB*, and *coa* were compared using real-time RT-PCR in WT (*S. aureus* NCTC8325), SX19 (the *rot* mutant), SX15 (the *agr* mutant), SX17 (the *rnaIII* mutant), RN6911 (NCTC8325-4 *agr* mutant), and SX21 (RN6911 *rot* mutant). The strains were grown in LB medium to different growth phases, and the transcriptional comparison of *map*, *efb*, *fnbA*, *fnbB*, and *coa* in the strains that were grown to an OD_{600} of 1, 2, and 4 was shown in **a–c**, respectively. The real-time RT-PCR assays were repeated four times with similar results. *Error bars* indicate SD
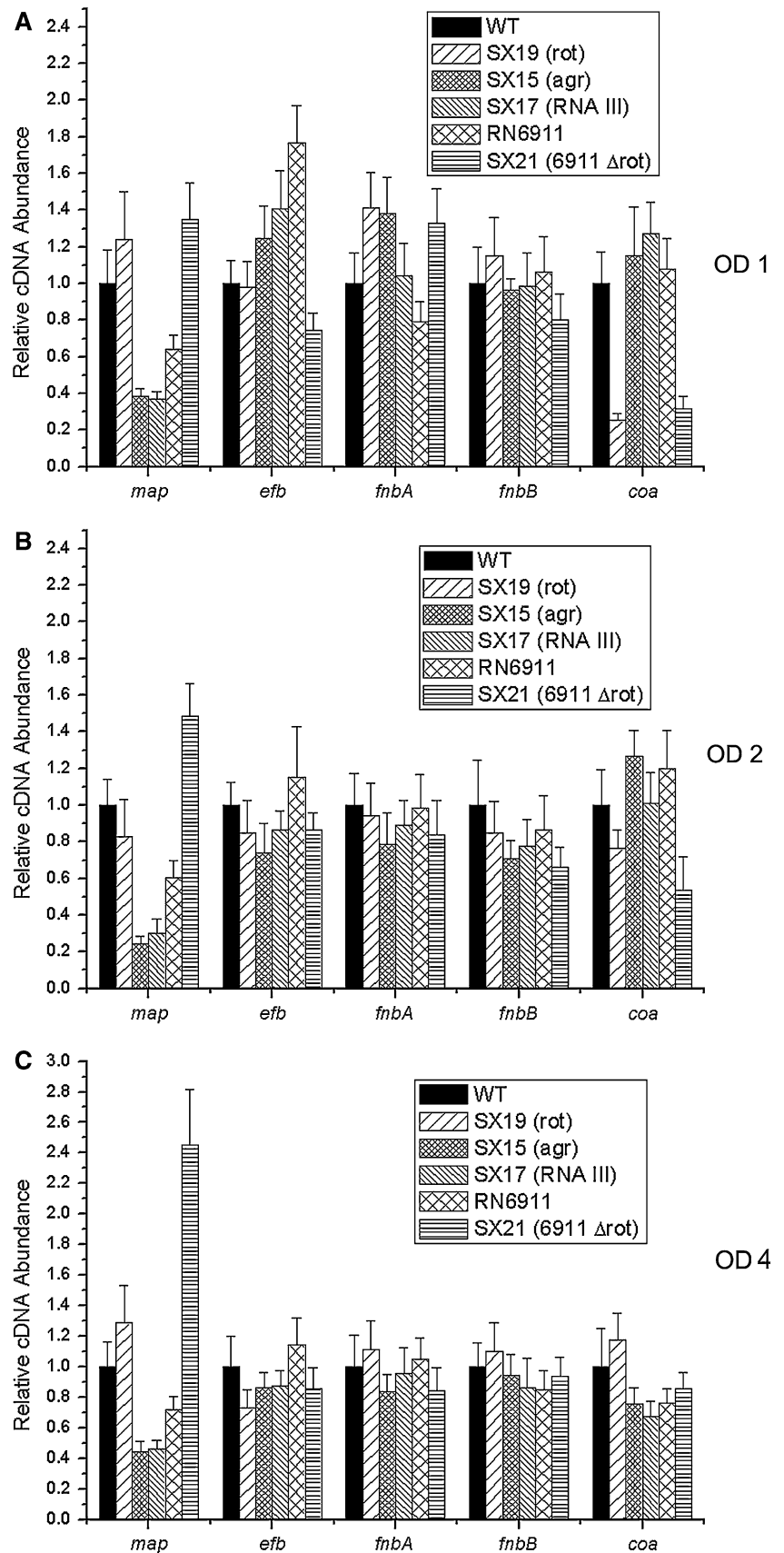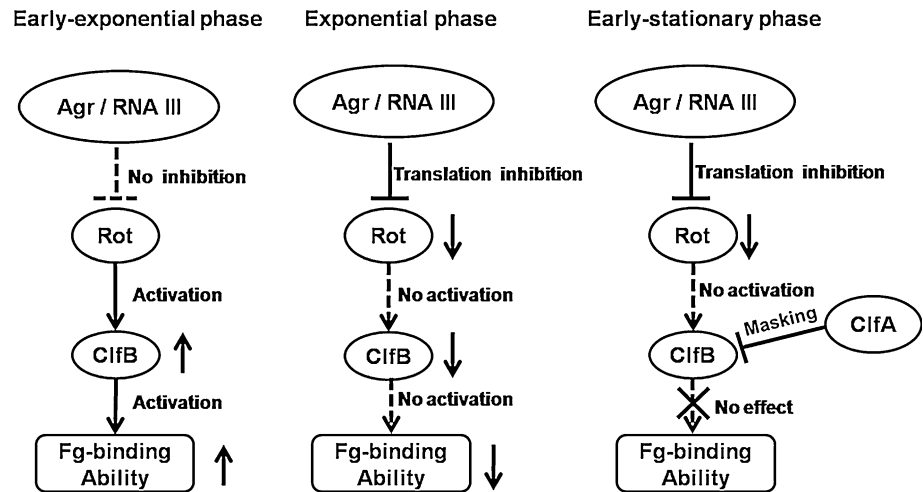
early-exponential phase but this activation is not apparent once bacterial cells enter exponential phase. This is because when the cells reach the exponential phase, the Agr quorum-sensing system starts to function and the abundance of RNAIII increases largely. A previous study has shown that RNAIII can inhibit translation of Rot mRNA by a limited number of base pairings [26, 27]. Therefore, the protein level of Rot decreases significantly and the activation of bacterial Fg-binding ability decreases accordingly. This also indicates that the Agr system indirectly inhibits bacterial Fg-binding ability (Fig. 5).

The proteins that can bind to host Fg are mainly ClfA and ClfB. It has not been previously reported whether Rot can regulate *clfA/B* expression. Previous studies revealed that the transcription of *clfA* was positively influenced by sigma factor B [44–46], but this influence was weakened in *S. aureus* NCTC8325 because of the deficiency of sigma factor B. Moreover, our results showed that the transcription of *clfA* is not regulated by Agr or Rot in *S. aureus* NCTC8325. Thus, ClfB is quite probably the target protein regulated by Agr and Rot to influence bacterial Fg-binding ability. According to a previous study, ClfB was shown to promote the clumping of exponential phase cells in a solution of Fg as well as the adherence of exponential phase bacterial cells to immobilized Fg in vitro. It was shown to bind to Fg and to contribute to the Fg-binding activity of *S. aureus* Newman cells from exponential phase cultures but not of cells from stationary phase cultures [11]. The Fg-binding ability of ClfB is masked by ClfA when the cells enter the post-exponential phase [11, 48]. Our results indicated that the mRNA transcript level of *clfB* was always much higher than that of *clfA*, suggesting that ClfB might mainly undertake the mission of Fg-binding before its binding ability is masked by ClfA. Furthermore, our data showed that Rot can activate the transcription of *clfB* during the early-exponential phase, probably through direct

binding of Rot to the promoter region of *clfB*. After entering the exponential phase, the translation of Rot is inhibited by RNAIII, thus blocking Rot-mediated transcriptional activation of *clfB*. Furthermore, the regulatory effect of Rot or the Agr system on *clfB* correlated with that on bacterial Fg-binding ability, indicating that Rot and the Agr system might affect bacterial Fg-binding ability mainly through regulating *clfB* transcription (Fig. 5). Additionally, there is another proof that can demonstrate this conclusion. As shown in Fig. 3b, when the bacterial cells entered the early-stationary phase ($OD_{600} = 4$), mutation of *agr* significantly increased *clfB* transcription, whereas the regulatory effect of Agr on bacterial Fg-binding ability was not obvious (Fig. 1c). This leads us to suggest that although Agr is still regulating the transcription of *clfB* via Rot during this growth phase, the protein activity of ClfB is masked by ClfA, which continues to mediate the interactions between Fg and bacterial cells from stationary phase cultures. Therefore, the regulatory effect of Agr on *clfB* cannot result in any obvious phenotypic change. This also indicates that the regulation by Agr and Rot of bacterial Fg-binding ability is mainly through regulating the transcript level of *clfB*.

Several secretory proteins (coagulase, FnbpA, FnbpB, Efb, and Map) also bind to Fg. However, compared with ClfA and ClfB, the Fg-binding abilities of these proteins are weaker. According to our results, the transcription of these genes (*efb*, *fnbA*, and *fnbB*) was not affected by the Agr system or Rot. Interestingly, both Rot and Agr can affect the transcription of *map* but this influence is contrary to the regulatory effect of Rot and Agr on *clfB* transcription.

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